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IN SILICO FUNCTIONAL ANNOTATION AND STRUCTURAL MODELING OF THE HYPOTHETICAL PROTEINS OF *HALOBACTERIUM SALINARUM* NRC-1

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ABSTRACT

Six hypothetical proteins of *Halobacterium salinarum* NRC-1 VNG0546c, VNG2021c, VNG2549c, VNG0683c, VNG2351c and VNG1475c were studied. Expasy's ProtParam study of physico-chemical properties showed that these halophilic proteins are acidic in character and stable in nature having high extinction coefficient ranging from 26164.9 to 57758.3 M 1 cm–1, high aliphatic index (except in VNG221c) and low Grand Average hydropathy value. Secondary structure by SOPMA predicted alpha helix dominates among secondary structure elements followed by random coil, extended strand and beta turns for all sequences (except VNG1475c). Functional characterization was performed by prediction of motifs and conserved domains. Three dimensional structures were developed for these halophilic proteins. The modeling of the three dimensional structure of these proteins was done by PHYRE 2 sever. Protein structure checking tools PROCHECK and ProSA were used for validating the modeled structures.

KEYWORDS: Hypothetical proteins, *Halobacterium salinarum* NRC-1, Isoelectric point, PHYRE 2.

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INTRODUCTION

The salt loving microorganisms "halobacteria" are the halophilic archaea which inhabit hyper saline environments such as salt lakes. These belong to the *Halobacteriaceae* family that is the only family in the *Halobacteriales* order[1] under the phylum *Euryarchaeota*. *Halobacterium salinarum* NRC-1[2,3] a gram negative halophilic archaeon is a marine and obligate aerobe belonging to the family *Halobacteriaceae.* Since the genome sequencing has been started, a large number of genomes have been sequenced and determined. These sequence studies provide information's that help in understanding the physiology and machinery which the organisms possess for its survival and adaptation in nature, even harsh conditions. The genome sequencing of *Halobacterium salinarum* NRC-1[2, 3] has also been done which contributes in studying the features of this halophilic archaea. The genome of *Halobacterium* consists of a large chromosome (2,014-kb) in conjunction with two mini chromosomes (pNRC200 of size 365 kb and pNRC100 of size 191 kb) which encodes 2,630 putative protein genes[2,3]. Nevertheless, greater part of the genome is still functionally unknown which results in a majority of genes remaining functionally unidentified and a large number of proteome designated as "hypothetical proteins". A significant fraction of the genes encodes 'conserved hypothetical' proteins in the sequenced genomes which are found from a number of phylogenetic lineages in organisms but have been functionally uncharacterized[4]. These proteins are not experimentally predicted but known only from a gene's nucleic acid sequence resulting in protein sequences functionally and structurally unknown. With the help of computational biology the structure modeling and functional annotation of these conserved hypothetical proteins can endow with more opportunity for studying their role in the machinery of the organism and mounting the probability of their usage in near future. Halobacteria is not a bacterium but belongs to the domain archaea[5] commonly found in the water containing a high content of salt. Because of ether linked lipids, a particular archaeal characteristic the halobacteria distinguishes from halophilic bacteria[6]. They exhibit similar features to eukaryotes transcription mechanism and translation as well as prokaryotes creating another domain archaea[7]. Halophilic microbes produce stable enzymes which have the ability to function under high concentrations of salt at which most proteins either precipitate or denature. Computational tools provide researchers an opportunity to identify the physicochemical properties, and to characterize the functional and structural properties of the proteins. The experimental methods to characterize the proteins of diverse organisms have a major drawback of the involving long time frame and high expense. The amino acid sequence of the protein provides major of the information necessary for determining and characterizing the protein's function, physical and chemical properties. So, enormous computational tools available from different sources can assist in the investigation of structures of proteins. Functional characterization is done by identification of functional motifs and domains. The present paper is an in silico analysis and homology modeling studies of the six conserved hypothetical proteins of the *Halobacterium salinarum* NRC-1[2,3] whose three dimensional structures are not yet available in the public databases and are functionally unknown.

MATERIALS AND METHODS

Protein sequence retrieval

The protein sequences of six halophilic proteins were retrieved from Genbank[8] at National Centre for Biotechnology Information (NCBI)[9] which is a public domain Knowledgebase database.

Protein sequence analysis. The physico-chemical characterization of the protein was done by using the Expasy's ProtParam server[10]. The parameters computed by the tool are theoretical isoelectric point (pI), molecular weight, total number of positive

(+R) and negative (–R) residues, extinction coefficient[11], instability index[12], aliphatic index[13] and grand average hydropathy (GRAVY)[14]. Table 1 shows the results of sequence analysis.

Secondary structure prediction

SOPMA[15] was employed for computing and analyzing the secondary structural features of the halophilic protein sequences considered for this study SOPMA correctly predicts 69.5% of amino acids for a state description of the secondary structure prediction. The secondary structure indicates whether a given amino acid lies in a helix, turn, coil or strand. The default parameters were used for prediction.

Functional annotation and characterization of the proteins

For the functional annotation, conserved domains were detected in the halophilic proteins using sequence similarity search with close orthologous family members available in various protein databases using the web-tools. Four bioinformatics web tools used were CDD-BLAST[16] (http://www.ncbi.nlm.nih.gov/BLAST/), INTERPROSCAN[17] (http://www.abi.ac.uk/interpro), Pfam[18] (http://www.pfam.sanger.ac.uk/) and CDART[19] (www.ncbi.nlm.nih.gov/Structure/) which shows the ability to search the defined conserved domains and motifs in the sequences and guide in the classification of conserved protein families. Motifs are specific regions, around 10 to 20 amino acid sequences in length arising because of conserved in both structure and sequence during evolution and which are involved in the biological function of a group of proteins.

3D model building of proteins

The three dimensional modeling of the protein was performed by PHYRE 2 server[20]. The Phyre server utilizes a library of known protein structures taken from the Structural Classification of Proteins (SCOP) database and increased with newer depositions in the Protein Data Bank (PDB).

Phyre2 utilizes fold library (containing the profiles of the structures of the sequences which is made by scanning against a non redundant database) and the hidden Markov models alignment using HHsearch[21] to considerably improve alignment accuracy and detection rate. Phyre2 also includes Poing[22] which is a new ab initio folding simulation to model regions of the proteins in case of no detectable homology to known structures. The 3D models of the top ten highest scoring alignments are performed. A loop library and reconstruction procedure are also used for the repairing of missing regions and inserted regions due to insertions and deletions and side chains are positioned on the model using a fast graph-based algorithm and side chain rotamer library. In this study the model created with template showing the maximum percent identity and confidence level are chosen (Table 5).

MODEL EVALUATION

Once 3D models were generated, the overall stereo chemical property of the proteins was assessed by Ramchandran plot analysis[23]. The validation for structure models obtained from PHYRE 2 was performed by using PROCHECK[24] and ProSA[25]. A protein structure analysis program PROCHECK[24] available at the Joint Centre for Structural Genomics, Bioinformatics core at University of California, San Diego (USCD) was employed in validation of protein structure and models by verifying the parameters like Ramachandran plot quality, peptide bond planarity and over-all G factor. ProSA[25] web Z score was used for structural evaluation of the modeled proteins. ProSA[25] tool was widely used to verify 3D models of protein structures for possible errors. Furthermore, structure analysis and visualization of generated models was performed using UCSF Chimera 1.8[26].

RESULTS AND DISCUSSION

Expasy's ProtParam tool's computed Parameters are represented in Table I. The theoretical isoelectric point (pI) is the pH at which the surface of protein is covered with charge but net charge of protein is zero and the proteins are stable. It is useful because at pI, solubility is least and mobility in an electro focusing system is zero. The computed pI

value of all the six halophilic proteins are less than 7 (pI<7) indicating that these halophilic proteins are acidic in character. Expasy's ProtParam[10] computes the extinction coefficient at 280 nm wavelength which is the most favored as the proteins absorb light strongly at 280 nm. Extinction coefficient of halophilic proteins at 280 nm is ranging from 26164.9 to 57758.3 M 1 cm^{-1} in order of the concentration of Cys, Trp and Tyr residues. The high extinction coefficient of proteins indicates the presence of high levels of Cys, Trp and Tyr amino acids. The computed extinction coefficients are useful in the quantitative study of protein-protein and protein–ligand interactions in the solution. The instability index value for the halophilic hypothetical proteins is found to be ranging from 24.49 to 36.99. As the protein with instability index[12] (II) smaller than 40 is predicted as stable whereas protein may be unstable above a value 40, the result classified all six halophilic proteins as stable protein (Table 1). Halophilic protein sequences have an aliphatic index ranging from 61.73–94.40. The very high aliphatic index of all halophilic protein sequences indicates that these proteins may be stable for a wide temperature range[13]. The lower thermal stability of VNG2021c indicates a more flexible structure when compared to other halophilic proteins. Grand Average hydropathy (GRAVY) indices of halophilic proteins are ranging from - 0.011 to -0585. The low range Grand Average hydropathy of values indicates the possibility of better interaction with water.

TABLE 1
Physico-chemical Parameters predicted using Expasy ProtParam tool

proteins	Sequence length	Molecular weight	pI	-R	+R	Extinction coefficient	Instability index	Aliphatic Index	GRAVY
VNG1475c	551	57758.3	4.28	76	35	28310	35.47	80.45	-0.275
VNG2549c	376	40557.3	4.10	65	22	35870	24.96	81.28	-0.198
VNG2021c	492	55036.5	4.20	115	37	70820	28.84	61.73	-0.585
VNG0683c	263	28083.0	4.38	44	20	42400	24.49	76.88	-0.286
VNG2351c	285	31051.5	4.09	60	26	19940	33.43	91.58	-0.272
VNG0546c	250	26164.9	4.03	54	14	11585	36.99	94.40	-0.011

The secondary structures of halophilic hypothetical proteins are predicted by SOPMA[15] (Self Optimized Prediction Method with Alignment) show that alpha helix dominates among secondary structure elements followed by random coil, extended strand and beta turns for all sequences except VNG1475c. In protein VNG1475c the random coil dominates followed by extended strand and alpha helix. SOPMA predicted secondary structure features are represented in Table 2.

TABLE 2
secondary structure prediction by SOPMA server

PROTEIN → / Secondary structure ↓	VNG1475c	VNG2549c	VNG2021c	VNG0683c	VNG2351c	VNG0546c
3_{10} helix	3.45%	46.81%	43.50%	37.64%	38.60%	41.20%
Pi helix	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Beta bridge	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Extended strand	36.12%	10.90%	15.24%	15.97%	20.70%	20.40%
Beta turn	4.90	5.85%	4.67%	7.98%	5.96%	8.00%
Bend region	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Random coil	55.54%	36.44%	36.59%	38.40%	34.74%	30.40%
Ambigous states	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Other states	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

Four online web servers (NCBI CDD blast, Pfam, INTERPROSCAN and CDART) find the conserved domains and motifs in the protein sequences to allocate the proteins in their significant families and to annotate their functional characteristics which are shown in the table 3.

TABLE 3
PROBABLE CONSERVED DOMAINS PRESENT IN HYPOTHETICAL PROTEINS OF Halobacterium salinarum NRC-1

server → / protein ↓	Pfam	CDD BLAST	INTERPROSCAN	CDART
VNG1475c	CARDB (Cell adhesion related domain)	NPCBM (novel putative carbohydrate binding module) - associated, NEW3 domain of alpha-galactosidase	APHP (acidic peptide-dependent hydrolases/peptidase) domain	NPCBM (novel putative carbohydrate binding module) - associated, NEW3 domain of alpha-galactosidase
VNG2549c	Periplasmic binding protein	Helical backbone metal receptor (TroA-like domain) (predicted to function as initial receptors in the ABC metal ion uptake)	ABC (ATP binding cassette) transporter periplasmic binding domain	Helical backbone metal receptor (TroA-like domain)
VNG2021c	Chlorite dismutase enzyme	putative heme peroxidase	Chlorite dismutase Enzyme(32-240) Antibiotic biosynthesis monooxygenase (407-477)	Chlorite dismutase Enzyme(32-240) Antibiotic biosynthesis monooxygenase (407-477)
VNG0683c	DeoC/LacD family aldolase	Class I fructose-1,6-bisphosphate (FBP) aldolases of the archaeal type (DhnA homologs)	DeoC/LacD family aldolase	Class I aldolases superfamily
VNG2351c	CBS (Cystathionine-beta synthase) domain	CBS (Cystathionine-beta synthase) domain	CBS (cystathionine-beta-synthase) domains	CBS (cystathionine-beta-synthase) domains
VNG0546c	PAC2 (Proteasome assembly chaperon)	Uncharacterized protein (ATP-grasp superfamily)	PAC2 (Proteasome assembly chaperon)	PAC2 (Proteasome assembly chaperon)

Out of six halophilic protein sequences, three dimensional structures are modeled for only four proteins which are VNG0546c, VNG2021c, VNG0683c and VNG2351c. The two proteins (VNG1457c and VNG2549c) could not be modeled because of the lack of the appropriate template for their sequences. The modeling of the three dimensional structure of the protein is performed by PHYRE 2 homology modeling program. The template used for homology modeling, confidence limit and percent identity by PHYRE 2 server is shown in the table 4.

TABLE 4
Template for the query protein, confidence and percent identity as predicted by PHYRE 2

Protein	Template	Confidence	% identity
VNG0546c	3VRO	100%	39%
VNG0683c	1OJX	100%	49%
VNG2021c	1TOT	100%	48%
VNG2351c	2YZQ	99.97%	31%

The template selected for protein VNG0546c is 3VRO

TABLE 6
ProSA web results

Protein	query	template
VNG0546c	-6.1	-7.04
VNG0683c	-8.6	-10.36
VNG2021c	-7.74	-8.28
VNG2351c	-7.16	-9.31

The evaluation of the predicted models generated by PHYRE 2 is shown in Figure 2. The parameters plotted by PROCHECK are Ramachandran plot quality, peptide bond planarity, over-all G factor, main chain hydrogen bond energy, Bad non bonded interactions and C-alpha chirality. The residues are classified according to its regions in the quadrangle in the Ramachandran plot analysis. The result revealed that the modeled structure for VNG0546c, VNG2021c, VNG2351c, VNG0683c has 88.4%, 93.6%, 80.0%, 87.9% and 92.3% residue respectively in allowed region. The main chain bond lengths and bond angles distribution were found to be within the limits for these proteins. The ramachandran plot analysis of the proteins by PROCHECK are given in Fig 5-8. Ramachandran plot results represent a good quality of the predicted models. Procheck results are shown in Table 5.

TABLE 5

RAMACHANDRAN PLOT analysis of structure predicted by PHYRE 2 using PROCHECK

protein → / Ramachandran plot ↓	VNG0546c	VNG2351c	VNG0683c	VNG2021c
Residues in the most Favored Region	88.4%	87.9%	92.3%	93.6%
Residues in additionally allowed region	10.6%	9.7 %	6.7%	6.4%
Residues in generously allowed region	1%	2.4%	0.5%	0.0%
Residues in disallowed region	0.0%	0.0%	0.5%	0.0%

The modeled structures of halophilic proteins are also validated by other structure verification server ProSA shown in Table 6. The z-score indicates overall model quality and measures the deviation of the total energy of the structure with respect to an energy distribution derived from random conformations[27]. The proximity of the Z score of the query structures with the experimentally derived structures confirms that the obtained models are of good quality and reliable. The validated structures of the proteins VNG0546c, VNG0683c, VNG2021c, VNG2351c are given in the Figures 1-4 based on the templates 3VRO (Crystal Structure Of Pyrococcus Furiosus Pbab, An Archaeal Proteasome Activator), 1OJX (Crystal Structure Of An Archaeal Fructose 1,6-Bisphosphate Aldolase), 1TOT (Crystallographic Structure Of A Putative Chlorite Dismutase), 2YZQ (Crystal Structure Of Uncharacterized Conserved Protein From Pyrococcus Horikoshii) respectively.

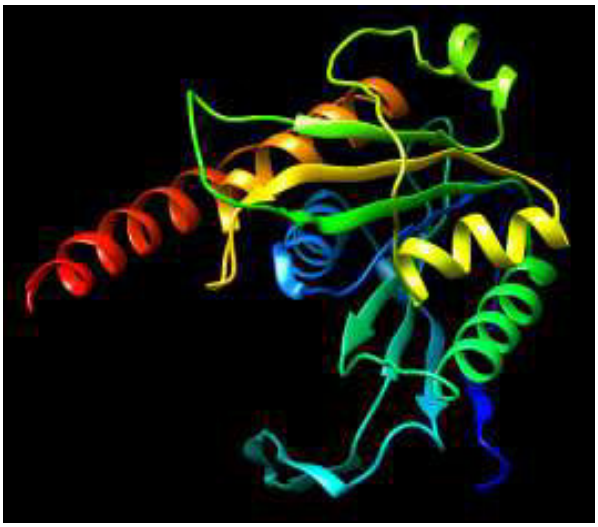

Figure 1

Structure of hypothetical protein VNG0546c predicted by PHYRE 2 using template 3VRO

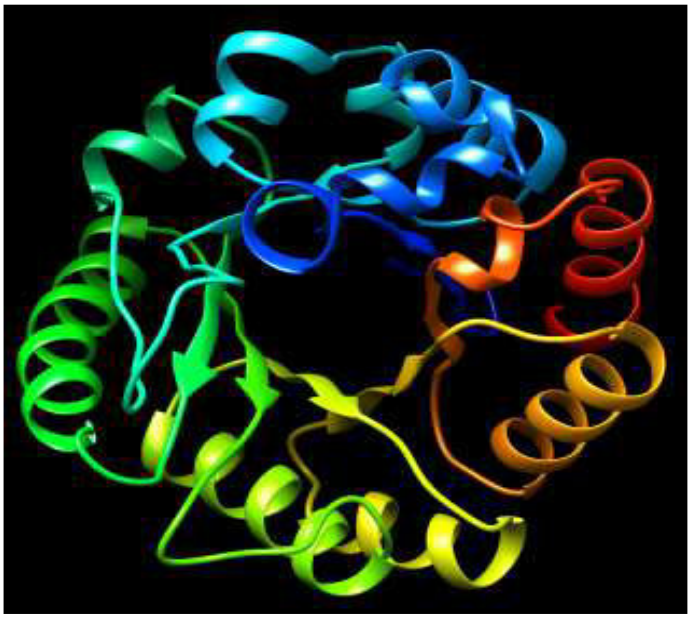

Figure 2
Structure of hypothetical protein VNG0683c predicted by PHYRE 2 using template 1OJX

Figure 3
Structure of hypothetical protein VNG2021c predicted by PHYRE 2 using template 1TOT

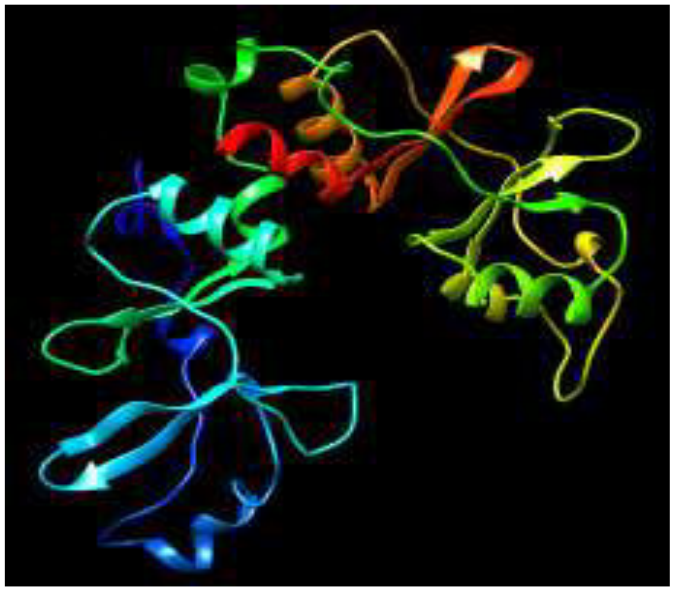

Figure 4
Structure of hypothetical protein VNG2351c predicted by PHYRE 2 using template 2YZQ

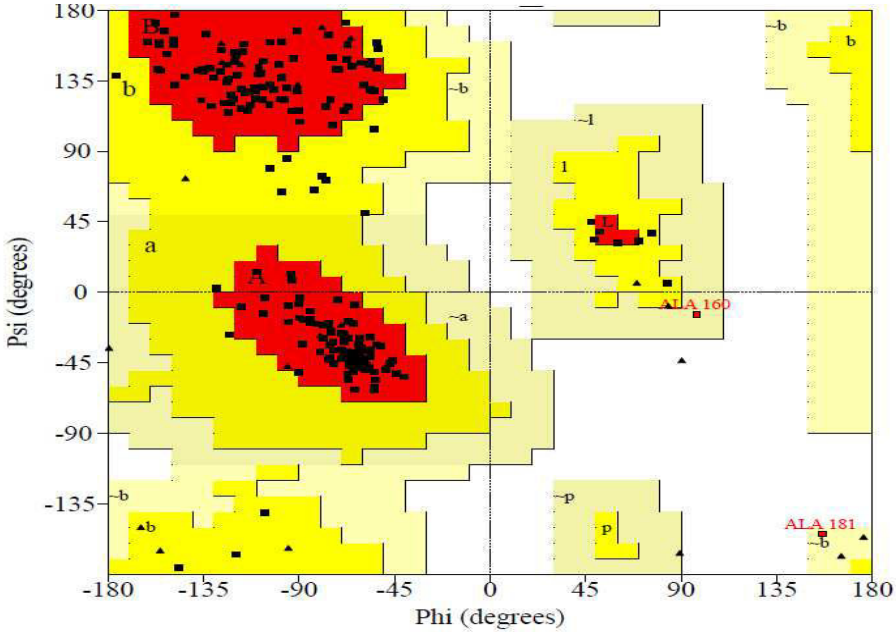

Figure 5
Ramachandran plot analysis of VNG0546c

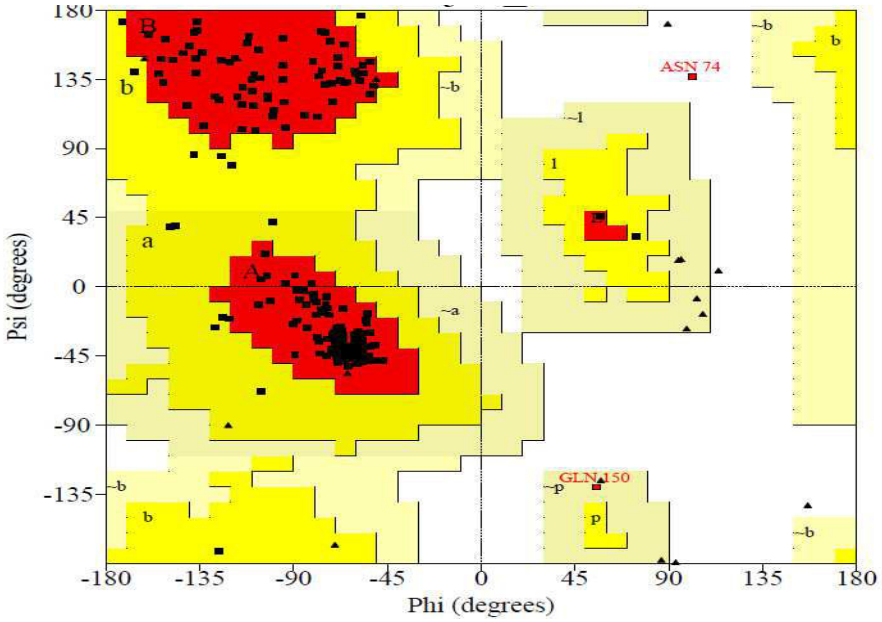

Figure 6
Ramachandran plot analysis of VNG0683c

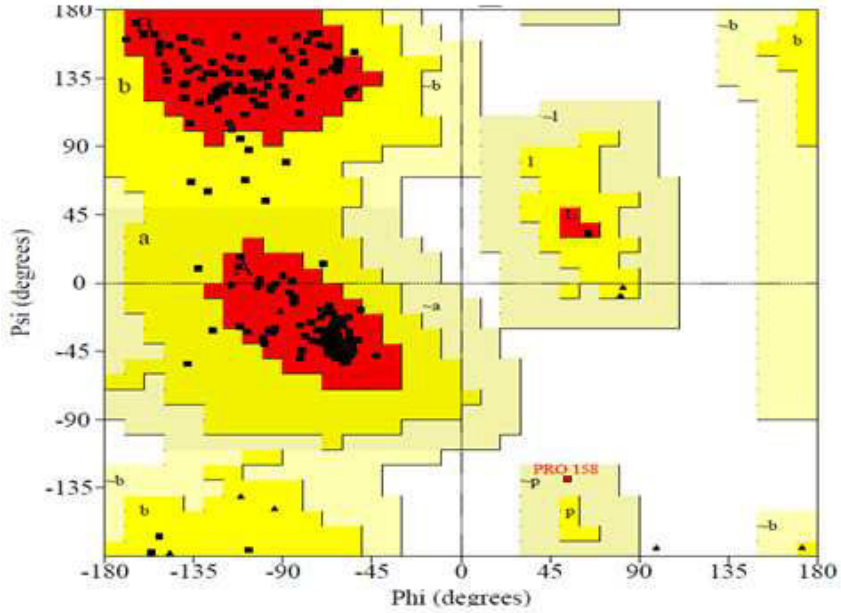

Figure 7
Ramachandran plot analysis of VNG2021c

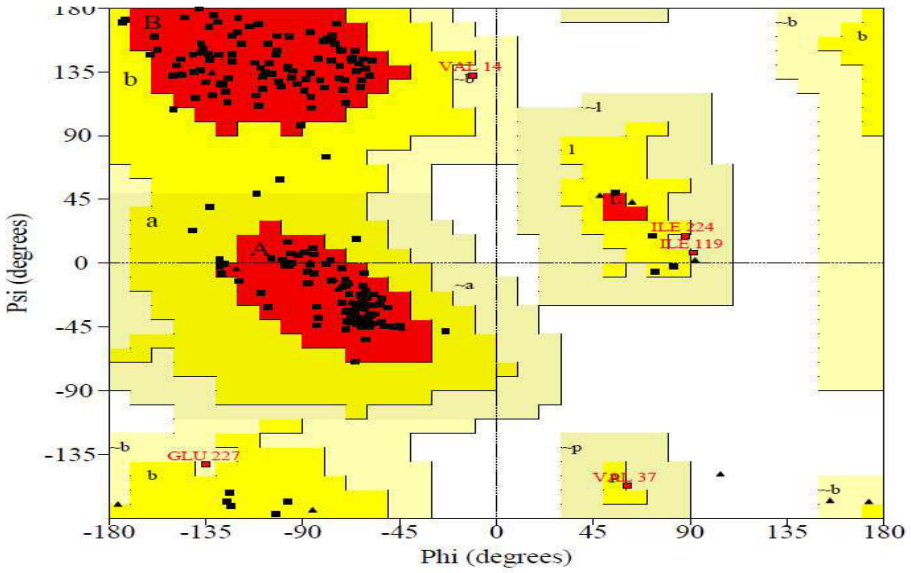

Figure 7
Ramachandran plot analysis of VNG2351c

CONCLUSION

Through this study some functional hypothetical proteins of *Halobacterium salinarum* NRC-1 by applying different parameters were studied along with structure prediction. It suggests that many uncharacterized proteins are available in the halophilic archea whose functional role is still vague in their machinery. Progress in genome sequencing, sequence databases and analysis tools have enhanced the methods to study inherent properties of the proteins and conclude functional interactions in the hypothetical proteins under study. Bioinformatics tools like CDD-BLAST, INTERPROSCAN, PFAM and CDART shows the capability to assign functions to the hypothetical proteins. PHYRE 2 server provide the three dimensional models of the hypothetical proteins with great accuracy will serve as a good foundation for functional analysis of experimentally derived crystal structures (as carried out for halophilic viruses[28]) and basis for exploration of their role in the archaeal domain.

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